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Effect of stress on heat shock protein levels, immune response and survival to fungal infection of *Mamestra brassicae* larvae

E.H. Richards [a,*], M.P. Dani [a], Y. Lu [a], T. Butt [b], R.J. Weaver [a]

[a] Fera, Sand Hutton, York YO41 1LZ, UK
[b] Department of Biosciences, College of Science, Swansea University, Singleton Park, Swansea SA2 8PP, UK

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ABSTRACT

Although the utilisation of fungal biological control agents to kill insect pests is desirable, it is known that the outcome of infection may be influenced by a number of criteria, including whether or not the target insect is stressed. In the current work, topical treatment of larvae of the lepidopteran pest, *Mamestra brassicae*, with conidia of *Beauveria bassiana*, followed by a heat stress (HS; 37 °C for 1 h) 48 h later, resulted in a similar level of larval survival to that occurring for no heat stress (No-HS), fungus-treated larvae. By contrast, when the HS was applied 24 h after fungal treatment, larval survival was significantly increased, indicating that the HS is protecting the larvae from *B. bassiana*. Similarly, exposure of larvae to a HS provided protection against *Metarhizium brunneum* (V275) at 48 h (but not 24 h) after fungal treatment.

To elucidate the mechanism(s) that might contribute to HS-induced increases in larval survival against fungal infection, the effects of a HS on key cellular and humoral immune responses and on the level of selected heat shock proteins (HSP) were assessed. When larvae were kept under control (No HS) conditions, there was no significant difference in the haemocyte number per ml of haemolymph over a 24 h period. However, exposure of larvae to a HS, significantly increased the haemocyte density immediately after (t = 0 h) and 4 h after HS compared to the No HS controls, whilst it returned to control levels at t = 24 h. In addition, *in vitro* assays indicated that haemocytes harvested from larvae immediately after (0 h) and 4 h (but not 24 h) after a HS exhibited higher rates of phagocytosis of FITC-labelled *B. bassiana* conidia compared to haemocytes harvested from non-HS larvae. Interestingly, the HS did not appear to increase anti-fungal activity in larval plasma. Western blot analysis using antibodies which cross react with *Drosophila melanogaster* HSP, resulted in a relatively strong signal for HSP 70 and HSP 90 from extracts of 50,000 and 100,000 haemocytes, respectively, harvested from No-HS larvae. By contrast, for HSP 60, a lysate derived from 200,000 haemocytes resulted in a relatively weak signal. When larvae were exposed to a HS, the level of all three HSP increased compared to the No HS control 4 h and 16 h after the HS. However, 24 h after treatment, any heat stress-mediated increase in HSP levels was minimal and not consistently detected. Similar results were obtained when HSP 90, 70, and 60 levels were assessed in fat body harvested from heat stressed and non-heat stressed larvae. With regard to HSP 27, no signal was obtained even when a lysate from 200,000 haemocytes or three times the amount of fat body were processed, suggesting that the anti-HSP 27 antibody utilised does not cross-react with the *M. brassicae* HSP. The results suggest that a HS-mediated increase in haemocyte density and phagocytic activity, together with an upregulation of HSP 90 and 70, may contribute to increasing the survival of *M. brassicae* larvae treated with *B. bassiana* and *M. brunneum* (V275).

1. Introduction

There is an urgent need to find replacements for many chemical pesticides currently used to control insect pests of relevance to the agricultural, horticultural and forestry sectors. One approach focuses on the use of insect-specific biological control agents (BCA), including viruses, bacteria, and entomopathogenic fungi

Abbreviations: BCA, biological control agent; HS, heat stress; No HS, no heat stress; HSP, heat shock protein; Bb, *Beauveria bassiana*; CE, constant environment room; PDA, potato dextrose agar; PD, potato dextrose; DPBS, Dulbeccos's phosphate buffered saline; FITC, Fluorescein isothiocyanate; GLMM, Generalized Linear Mixed Model; EPF, entomopathogenic fungus.

* Corresponding author.
E-mail address: elaine.richards@fera.co.uk (E.H. Richards).

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(EPF) (see Lacey et al., 2015 and references therein). Different strategies may employ these BCA on their own, or as part of an integrated pest management regime (Butt et al., 2001; Sandhu et al., 2012; Lacey et al., 2015). With regard to EPF, strains of *Beauveria spp.* and *Metarhizium spp.* are often utilised. Both of these fungi have a broad host range and have been used to control a variety of crop pests, including European corn borer, termites, whitefly, green leafhoppers, locusts, grasshoppers, etc. (Butt et al., 2001; Sandhu et al., 2012; Lacey et al., 2015). They also show potential for controlling insect pests of trees, including the Asian longhorn beetle (Dubois et al., 2004; Shanley et al., 2009) and the pine processionary moth (Er et al., 2007; Sevim et al., 2010).

Despite the desire to use EPF to control insect pests, it is generally acknowledged that the interaction of a fungus with any given insect host/target is complex and that the outcome of infection (death of the insect pest or the EPF) may be influenced by a variety of factors. For instance, apart from evolving physical barriers to prevent cuticular penetration by the fungus (Ortiz-Urquiza and Keyhani, 2013), a major response of an insect to fungal infection is the mobilisation of haemocyte-mediated immune responses (including phagocytosis and encapsulation), and humoral immune responses (including the upregulation of anti-fungal molecules, phenoloxidase activity, reactive oxygen species, etc.) in order to protect itself from the fungus (Lemaitre and Hoffmann, 2007; Stokes et al., 2015; Butt et al., 2016). Similarly, fungi have evolved a number of strategies that enable them to infect and disable insects, including the production of molecules designed to suppress insect immune responses (Bulet and Stocklin, 2005; Ortiz-Urquiza and Keyhani, 2013; Butt et al., 2016).

In view of this, an active area of research is focussed on elucidating the molecular mechanisms that influence the efficacy of fungal BCA for any given pest insect. For instance, the efficacy of EPF may be improved by increasing their virulence through genetic modification (Wang and St. Leger, 2007; St. Leger and Wang, 2010), by suppressing relevant immune responses in the target pest insect (Dean et al., 2002; Richards et al., 2011, 2013), and/or by utilising other BCA that act synergistically with the fungal BCA (Ansari et al., 2008). By contrast, other stresses may decrease the efficacy of EPF. For example, in the wax moth, *Galleria mellonella* (an insect that lives communally in bee hives, where temperatures may reach 40 °C), it was demonstrated that exposure of the larvae to a heat shock of 43 °C for 15 min after natural infection with *B. bassiana*, positively affected their survival by extending the life time compared to larvae left at a culturing temperature of 28 °C (Wojda et al., 2009). Moreover, it was shown that the increase in survival was not due to a deleterious effect of heat shock on the fungus as similar results were obtained when larvae were given a heat shock first and then injected with *B. bassiana*. Interestingly, the heat stress (in conjunction with fungal infection), also increased the level of certain anti-microbial peptides in the haemolymph, and it was concluded that this likely accounted for the increased survival rate of the heat shocked larvae (Wojda et al., 2009). These results are corroborated and extended by studies that demonstrate that exposure of *G. mellonella* to a mild physical stress (shaking) and/or thermal stress resulted in short-term immune priming, which correlates with protection against infection by *Candida albicans* and *Aspergillus fumigatus* (Mowlds and Kavanagh, 2008; Mowlds et al., 2008; Browne et al., 2014).

The molecular mechanisms or pathways activated by heat shock or physical stress and how these culminate in increases in insect immune responses and survival, are not clear at present. Although, in *G. mellonella*, it has been hypothesised that stress-induced heat shock protein (HSP) 90 and/or HSP 90 derivatives may play a role (Wojda and Jakubowicz, 2007; Dubovskiy et al., 2013). HSP 90 is one of several HSP families that are grouped according to molecular weight (*e.g.* HSP 90, 70, 60, and the small HSP) (Parsell and Lindquist, 1993; Sun and MacRae, 2005; Richter et al., 2010). HSP are present in all cells in all forms of life. Under normal (unstressed) conditions, they function primarily as molecular chaperones and ensure the proper folding of nascent polypeptides. Following cellular stress, the appearance of denatured proteins and polypeptides stimulates an upregulation in gene expression of HSP, such that their level within the cell increases markedly. In addition to being induced by heat shock, HSP may also be up-regulated in response to a variety of stresses. In insects, such stresses may include diapause, anoxia, desiccation, different developmental stages, ageing, and exposure of insects to UV radiation, drought, oxidation, parasitoid envenomation, and a wide range of chemicals and contaminants (including heavy metals and ethanol) (*e.g.* Sonoda et al., 2007; Shim et al., 2008; Lopez-Martinez et al., 2009; Nguyen et al., 2009; Zhang and Denlinger, 2010; Michaud et al., 2011; Tower, 2011; Zhao and Jones, 2012; Kim et al., 2015). The hypothesis that *G. mellonella* HSP 90 and/or its derivatives stimulate immune responses and contribute to survival of the larvae against pathogens (Wojda and Jakubowicz, 2007; Wojda et al., 2009; Dubovskiy et al., 2013) is supported by studies in other insects. For instance, heat shock has also been shown to restrict virus infection in *Drosophila melanogaster* (Merkling et al., 2015), whereas in *Spodoptera frugiperda* Sf9 cells, induced and cognate HSP 70s were found at high levels in cells infected with *Autographa californica multiple nucleopolyhedrovirus* (Lyupina et al., 2011). In the red flour beetle, *Tribolium castaneum*, injection with crude lipopolysaccharides (LPS) induced strong expression of HSP mRNA transcripts (Altincicek et al., 2008). Also, eicosanoids have been shown to mediate small HSP gene response to biotic stress (including virus particles and *B. bassiana*) (Zhang et al., 2015a,b). These studies and others, suggest that in insects, the stress and immune responses are interlinked possibly sharing certain signal transduction pathways (Altincicek et al., 2008; Adamo, 2008; Wojda and Taszlow, 2013; Eggert et al., 2015; Zhang et al., 2015a,b). Moreover, physical stress can induce HSP and/or immune responses in other invertebrates (*e.g.* Singh and Aballay, 2006; Malagoli et al., 2007), whilst over the last two decades or so, a significant role for HSP in the immune system of mammals has emerged (Binder, 2014). This work raises the possibility that (under certain circumstances), RNAi-mediated knockdown of key HSP genes in pest insects could lead to a state of immunosuppression, which would increase their susceptibility to BCAs, including insect-specific EPF.

In the current work, the major aim was to gain an insight into how larvae of the lepidopteran pest, *M. brassicae*, respond to stress at the molecular level and whether exposure of larvae to stress can alter their susceptibility to fungal BCAs. More specifically, in view of the work performed previously using *G. mellonella*, and because *M. brassicae* larvae do not usually live at such relatively high temperatures (up to 40 °C), the study sought to determine the effect of a non-lethal heat stress on *M. brassicae* haemocyte number, and on humoral and haemocyte-mediated immune responses. Utilising a proteomic approach, the effect of heat stress on the levels of (selected) HSP in two immunocompetent tissues, fat body and haemocytes, was also examined. In addition, the virulence of two *M. brunneum* strains (4556 and V275 [AKA Met 52]) and one *B. bassiana* strain were compared for efficacy against *M. brassicae* larvae, and then the two most virulent strains were utilised in bioassays to determine if heat treatment of the larvae affects their susceptibility to the EPF. It is envisaged that results gained using *M. brassicae* larvae will be applicable to other insect pest species, including pests of trees and forestry.